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Resistance to the Sterne strain of *B. anthracis*: phagocytic cell responses of resistant and susceptible mice

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Inflammatory responses were compared *in vivo*, and host phagocytic cell functions compared *in vitro*, of mice resistant (CBA/J) and susceptible (A/J) to lethal infection with the Sterne strain of *Bacillus anthracis*. Polymorphonuclear leukocyte (PMN) and macrophage responses at the initial site of infection were slower in A/J mice than in CBA/J mice. Whereas in A/J mice, the number of PMN ultimately responding to infection was equal to, or greater than, that in CBA/J mice, fewer macrophages accumulated. A/J mice failed to clear relatively low doses of the organisms and died. *In vitro*, chemotactic responses to both serum- and bacteria-derived attractants were similar for macrophages from A/J and CBA/J mice but were reduced for PMN from A/J mice. PMN and macrophages from the two mouse strains phagocytosed and killed spores *in vitro* to a similar extent, although killing by A/J PMN could be blocked by prior uptake of large numbers of killed spores. Thus susceptibility to lethal infection with Sterne strain correlated with the delayed influx (PMN) and reduced accumulation (macrophages) of phagocytes at the initial site of infection, but not with defective *in vitro* uptake or killing of spores.

Key words: Bacillus anthracis; Sterne; mice; resistance; anthrax; neutrophils; macrophages; phagocytic leukocytes.

Introduction

The pathogenesis of infection and the host response to *Bacillus anthracis*, the toxigenic and invasive etiologic agent of anthrax, are obscure. Fully virulent strains of *B. anthracis*, are encapsulated and produce lethal toxin and edema toxin.[1] Animal species differ in innate resistance to infection with virulent strains of *B. anthracis*.[2] The mechanisms responsible for host resistance to lethal infection with *B. anthracis* are not well understood. Inbred strains of mice are killed by low doses of toxigenic and encapsulated strains of *B. anthracis*; however, the mouse strains differ greatly in susceptibility to attenuated strains, such as the nonencapsulated, toxin-producing Sterne strain of *B. anthracis*.[3–5] Sterne is used widely as a live veterinary vaccine, since it protects domestic and laboratory animals against challenge with fully virulent

strains.[1,6-8] It appears to protect laboratory animals better against anthrax than do the cell-free vaccines used in humans; but it can cause morbidity and mortality.[4-6,8,9]

Resistance of mice to Sterne is linked to inheritance of a functional gene, *Hc*, encoding complement component 5 (C5).[10,11] C5-derived peptides (C5a/C5a des arg) are important anaphylatoxins and chemoattractants for macrophages and neutrophils during inflammation;[12-15] and they have several leukocyte-activating properties.[16-19] The role of C5 and the host leukocyte response in the control of Sterne infection is unknown; but susceptibilities to various other pathogens have been linked to C5 deficiency and diminished leukocyte inflammatory responses.[20-26]

The purpose of this study was to characterize the inflammatory responses of Sterne-resistant and -susceptible mice to live Sterne spores and other inflammatory agents. The inflammatory responses of these mice were examined *in situ* by a subcutaneous infection model and were quantitated by elicitation of peritoneal exudates. We also examined *in vitro* functions of phagocytic leukocytes (chemotaxis, phagocytosis, and spore killing) potentially associated with host resistance to lethal infection. A better understanding of the host response to Sterne is required to develop a safer and more efficacious vaccine against anthrax.

Results

In situ *study of inflammatory response to infection*

In our previous studies,[4,5] susceptibilities of mouse strains to lethal infection with Sterne were determined by subcutaneous inoculation of spores. In the present experiments, the time course of spore germination and the cellular response to infection with Sterne were evaluated *in situ*. Resistant (CBA/J) and susceptible (A/J) mice were inoculated subcutaneously with 5×10^5 or 1×10^7 cfu of Sterne. Both doses killed A/J mice ($LD_{50} = 2.5 \times 10^3$), whereas CBA/J mice survived ($LD_{50} = 2 \times 10^7$).[4] Mice infected with a lethal dose developed prominent subcutaneous edema in the upper foreleg and adjacent areas [Fig. 1(a)-(c)], as observed previously.[4,5]

Germinated bacilli were observed in the subcutaneous tissues by 2 h post-inoculation with 10^7 cfu (both mouse strains) and by 4 h after infection with 5×10^5 cfu (A/J mice, only). Spores and bacilli were observed both extracellularly and within phagocytes, and, in A/J mice, extracellular bacilli appeared to increase in number with time. Myriads of the bacteria were present, often in long chains, by 2 days (lower dose) or 1 day (higher dose) after infection [Fig. 1(a)-(d)]. Bacteria were detected in the subcutaneous tissues, underlying muscles, local lymph nodes, and intravascularly in A/J mice infected with 10^7 cfu [Fig. 1(a)-(d)]. In contrast, decreasing numbers of organisms were observed with time in CBA/J mice. We observed no organisms in tissue samples from CBA/J mice inoculated with the lower dose. In tissues from CBA/J mice given the higher dose, the number of bacilli decreased by 6 h, and none were seen >24 h after inoculation [Fig. 1(e)].

Polymorphonuclear leukocytes (PMN) were the major constituents of the early subcutaneous infiltrates of both strains of mice [Fig. 1(a)]. The number and type of inflammatory cells present did not change significantly after 6 h in A/J mice; and mild to severe necrosis of invaded tissues was present in A/J mice infected with 10^7 cfu. Most of the animals died between 24 and 30 h, and all were dead by 48 h post-inoculation. In specimens from CBA/J mice infected with 10^7 cfu, mixed infiltrates of PMN and macrophages were observed by 24 h, and the proportion of macrophages increased in specimens collected 48 and 72 h post-inoculation [Fig. 1(e)]. None of the mice died.
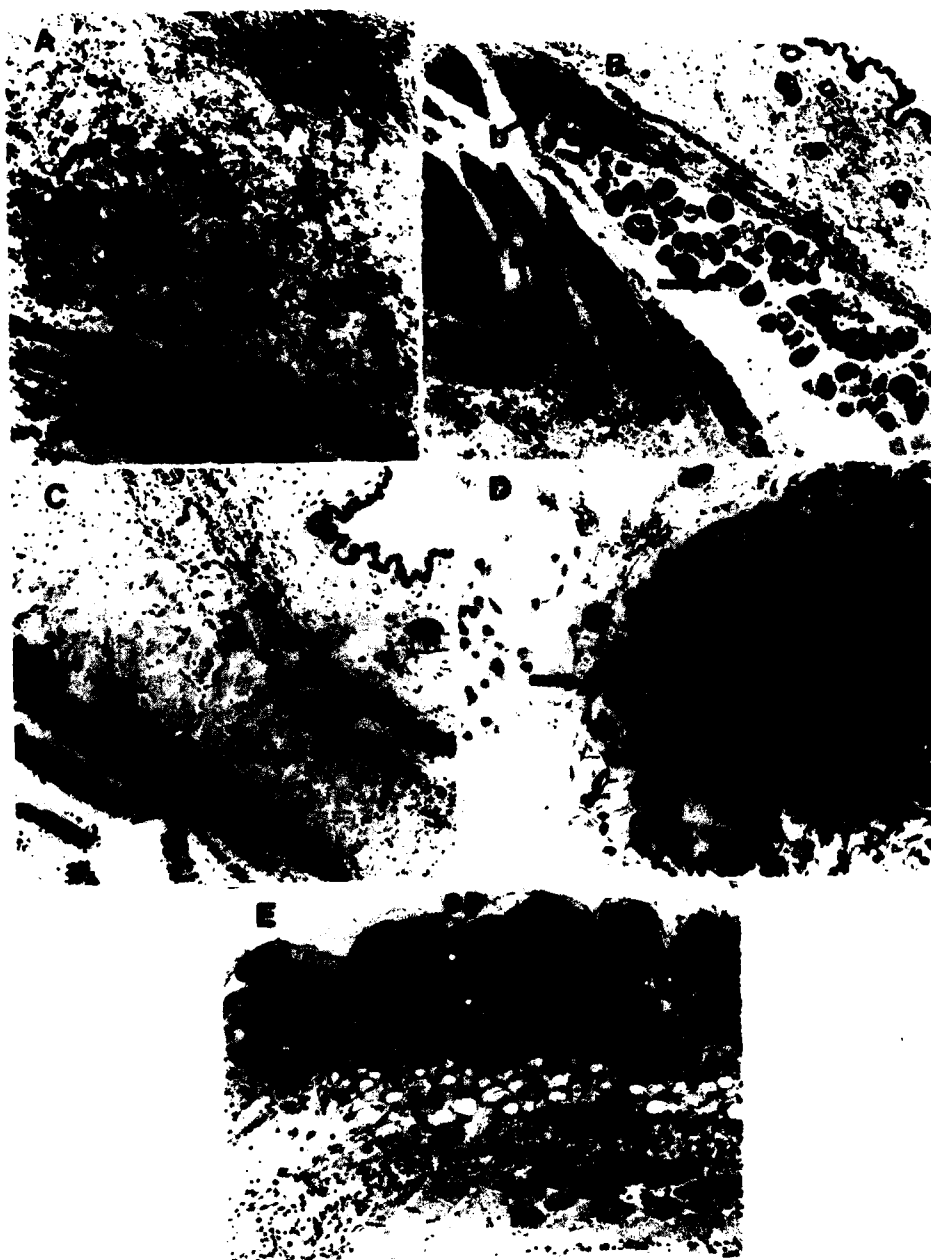
Fig. 1. Transverse sections from forelegs of mice inoculated s.c. with Sterne spores. Refer to part E for identification of normal structures. (A) PMN (p) appearing in subcutaneous tissue from A/J mouse collected 24 h after infection with 10^7 cfu spores; large numbers of bacilli (b) and subcutaneous edema (open areas) in dermis also present; HE, ×100. (B) Foreleg section from A/J mouse 30 h after infection with 10^7 cfu showing muscle necrosis (arrow), hemorrhage and edema, and extensive spread of bacilli (b) between muscle bundles (m); HE, ×100. (C) Same specimen as (B), stained with Giemsa to show the extensive spread of the bacteria; organisms also observed in blood vessels, bone marrow, and lymph node (not shown); ×100. (D) Same animal as B, axillary lymph node overgrown with bacilli (arrow); ×400. (E) Mixed subcutaneous infiltrate of macrophages and PMN in specimen obtained from CBA/J mouse 48 h after infection with 10^7 cfu; HE, ×100. The same specimen stained with Giemsa showed no detectable organisms and few tissue changes. ep—epidermis, d—dermis, sc—subcutaneous tissue, m—muscle (an axillary lymph node is not shown).

Quantitation of the in vivo *inflammatory response*

Inflammatory exudates were collected from A/J and CBA/J mice for quantitative comparisons of the cellular responses.

(i) Peritoneal exudates elicited with live spores. An intraperitoneal dose of 10^8 cfu spores was lethal to both CBA/J and A/J mice, but the relatively resistant CBA/J mice had a longer time to death. Almost 93% of A/J animals and only 38% of CBA/J mice were dead by 30 h post-inoculation. There were no differences in the number of PMN in exudates collected 4, 6 and 24 h after infection, although significantly fewer macrophages were observed in early samples from A/J mice (data not shown). However, macrophages represented a small proportion of the early inflammatory response, with levels not exceeding those present at the time of infection. As determined by microscopic counts, significantly fewer extracellular bacilli (but not fewer spores) were present in exudates of CBA/J mice than were in those of A/J mice from 1 to 24 h post-inoculation (data not shown).

The intraperitoneal inflammatory responses of mice to a lower dose of viable spores (2 to 3×10^6 cfu) were compared. This dose was not lethal for CBA/J mice but killed A/J mice by 3 days after inoculation; the intraperitoneal LD_{50} doses are 0.5 to 1×10^8 and 4×10^4 cfu, respectively. The PMN response of the A/J animals was greater than that of the CBA/J mice $\geqslant 6$ h after infection (Fig. 2). The numbers of macrophages observed at 0, 6 and 24 h post-inoculation were similar in both mouse strains. More macrophages were observed in CBA/J mice than in A/J mice at 48 h ($P < 0.05$). The CBA/J macrophage response continued to increase for several days after infection, whereas the A/J response peaked by 24 h post-inoculation, when the PMN response was decreasing (Fig. 2 and data not shown).

The early inflammatory responses to infection were studied in more detail in mice injected with 6×10^6 cfu of spores. Whereas samples from A/J and CBA/J mice obtained immediately after inoculation (0 h) contained comparable levels of leukocytes, significantly fewer PMN were present in exudates from A/J mice at 1 h and 2 h after infection (Fig. 3). Fewer macrophages were also found in exudates from A/J mice collected < 2 h after infection, as was observed with the largest dose of spores.

(ii) Peritoneal exudates elicited with sterile irritants and killed organisms. Starch- and thioglycollate-elicited exudates contained primarily PMN at 4 h and macrophages at 4 days after inoculation. A/J mice were deficient in both the PMN and macrophage responses to these irritants ($P < 0.0001$). Starch elicited twice the number of PMN in CBA/J mice, 13.1×10^6 (± 1.1), compared to A/J mice, 6.1×10^6 (± 0.7). It stimulated more than five times the number of macrophages in CBA/J mice, 10.8×10^6 (± 0.7), compared to A/J mice, 2.0×10^6 (± 0.9).

The PMN inflammatory response to 10^9 heat-killed Sterne bacilli reached a peak later in A/J (12 h) than in CBA/J mice (4 h) after injection. Subsequent samples collected through 48 h did not differ significantly in total PMN (data not shown). Compared to sterile irritants, killed bacilli (KB) elicited a relatively small macrophage response in both strains of mice ($< 3 \times 10^6$/specimen). As observed in response to 10^8 viable spores, exudates from CBA/J mice had more macrophages $\leqslant 48$ h ($P < 0.001$), but not 4 d, after inoculation with the KB (data not shown).

Fewer PMN were present in early A/J exudates elicited with $\leqslant 1 \times 10^8$ killed spores (KS) and collected 2 h after injection (Fig. 4). With the exception of the highest dose of KS, the number of PMN elicited in both strains was similar by 4 h (Fig. 4). These results suggest a slower response by A/J mice to KS, as observed with viable spores (Fig. 3) and KB. The early macrophage response was low in both strains, although
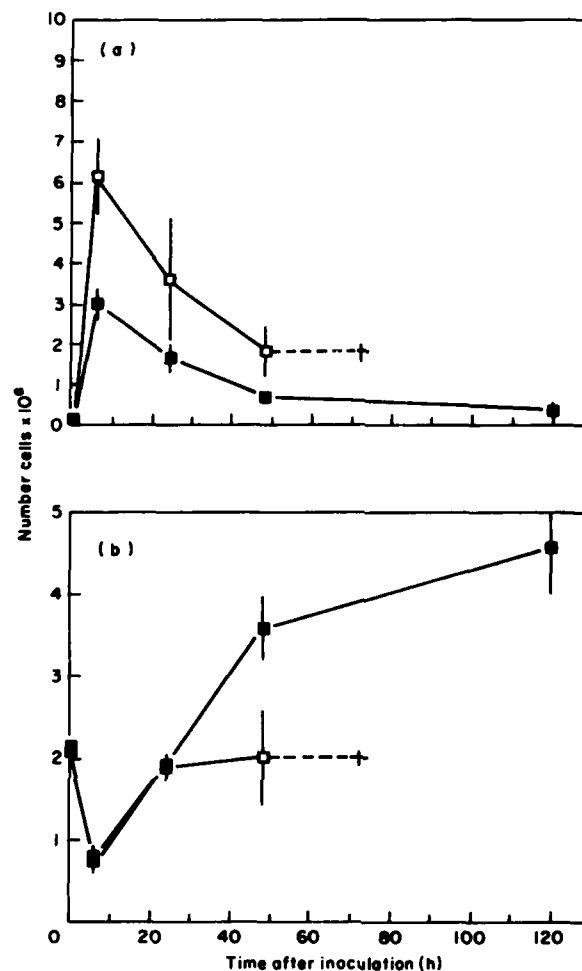
Fig. 2. Time course of peritoneal exudate response to infection with 2–3×10⁶ cfu Sterne spores. Exudate cells were counted, and the data are shown as mean and SEM (bars), six mice for each point. (a) Number of PMN were significantly greater ($P < 0.05$) in A/J specimens collected from 6 to 24 h PI. (b) Number of macrophages greater ($P < 0.05$) at 48 h PI in CBA/J exudates. ■ = CBA/J mice. □ = A/J mice. + = no survivors.

again greater at 2 h in specimens from CBA/J mice elicited with ≤1×10⁸ KS ($P ≤ 0.01$, data not shown).

(iii) Subcutaneous exudates elicited with KS and sterile irritants. Inflammatory exudates can also be elicited in subcutaneous sites which, unlike the peritoneal cavity, contain no resident leukocytes.[27] As we observed after intraperitoneal injection of sterile irritants, BioGel injected subcutaneously elicited a greater macrophage response in CBA/J mice than in A/J mice, with 7.3×10⁶ (±0.9) macrophages vs. 4.9×10⁶ (±0.5) macrophages per specimen, respectively ($P < 0.04$). A dose of 2×10⁷ KS induced a poor macrophage response in A/J mice, with 4.0×10⁴ (±2.0) macrophages per specimen. Significantly more macrophages, 7.2×10⁵ (±2.4), were elicited in the CBA/J mice, although most of the stimulation appeared to be attributable to the gelatin diluent which elicited 6.1×10⁵ (±3.4) macrophages/mouse. The subcutaneous
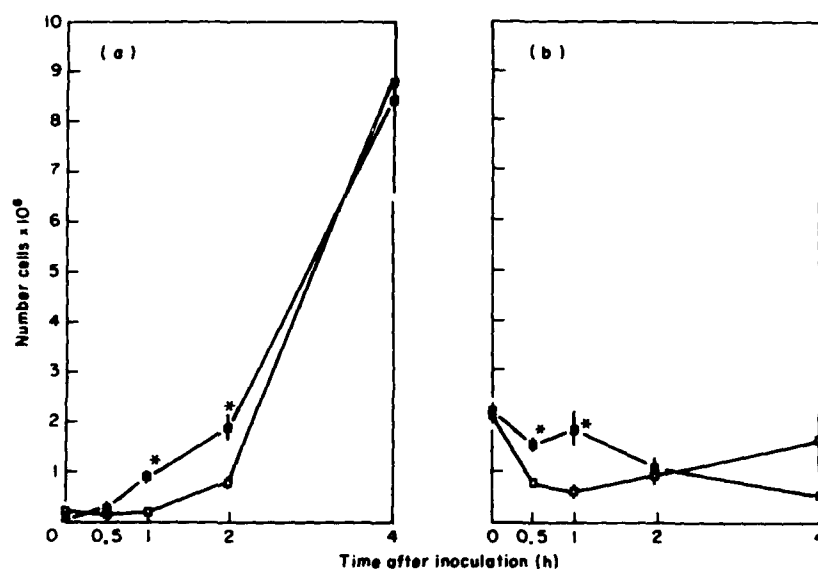
Fig. 3. Early peritoneal inflammatory responses to infection with 6×10^6 cfu Sterne spores. Exudate cells were counted and data shown as described in Fig. 2. Asterisks indicate CBA/J specimens containing significantly more leukocytes than A/J specimens. (a) Number of PMN was greater in CBA/J specimens collected at 1 h and 2 h p.i. ($P < 0.002$ and $P < 0.02$, respectively). (b) Number of macrophages were greater in CBA/J specimens collected at 0.5 h and 1 h p.i. ($P < 0.005$ for both).
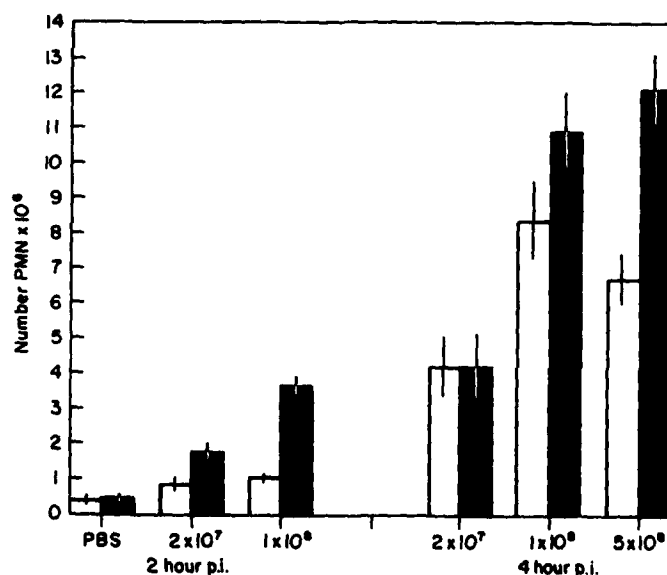

Fig. 4. Accumulation of peritoneal PMN 2 h and 4 h after inoculation with killed spores. A/J and CBA/J mice were injected intraperitoneally with one of three doses of killed spores (2×10^7, 1×10^8, or 5×10^8 spores) or PBS. Number of PMN harvested from CBA/J mice was significantly greater in specimens collected at 2 h (2×10^7, $P = 0.03$; 1×10^8, $P = 0.001$); but were greater only in the 4-h specimens elicited with 5×10^8 spores ($P < 0.002$). Data are mean and SEM (bars), where □ = A/J; and ■ = CBA/J.
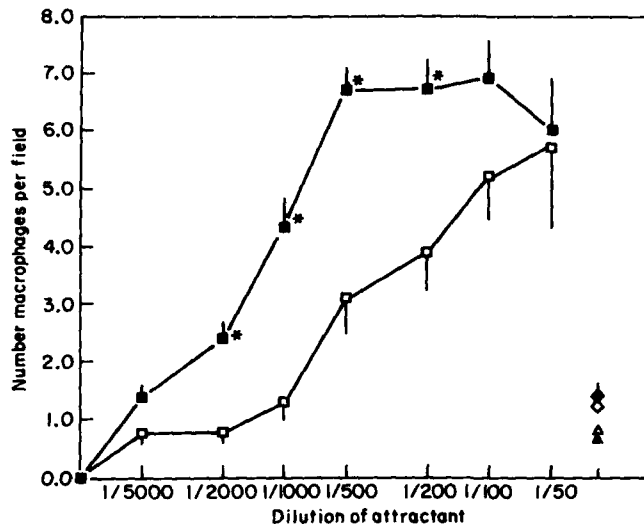
Fig. 5. Chemotactic responses of inflammatory macrophages to activated sera. Peritoneal macrophages were collected from mice inoculated intraperitoneally with starch 4.5 to 5 days previously. The migration of macrophages in response to CYAS (□, ■), to a 1/100 dilution of AYAS (◇, ◆), or to the GBSS+0.2% BSA diluent (△, ▲) was measured in a multiwell chemotaxis chamber after 120 min incubation at 37°C. Data are the mean number of macrophages per oil field ± SE, obtained from 3–4 wells in each of four experiments. Open symbols, A/J mice; solid symbols, CBA/J mice. *P < 0.002, compared to the corresponding A/J value.

macrophage responses correlated with the large intraperitoneal macrophage response to sterile irritants and small intraperitoneal response to KB.

Chemotaxis

We compared the *in vitro* chemotactic responses of phagocytic leukocytes from A/J and CBA/J mice.

(i) Activated serum attractant. Starch-elicited macrophages from both A/J and CBA/J mice responded chemotactically to activated normal serum from the C5-positive mouse strain, CBA/J. The macrophage did not differ significantly in their migration kinetics in response to a 1/100 dilution of yeast-activated (CYAS) or endotoxin-activated (EAMS) serum from CBA/J mice for periods up to 4 h (data not shown).

The effect of different concentrations of activated sera on the migration of starch-elicited macrophages is shown in Fig. 5. Chemotactic responses of macrophages from both strains exhibited a dose-dependent response to CYAS. A/J macrophages responded maximally to the lowest dilution (1/50) of attractant, whereas the CBA/J macrophage response peaked at dilutions of CYAS of ≤1/500. No differences in random migration (chemokinesis) were observed, and activated serum from C5-negative A/J mice had little activity for macrophages from either mouse strain (Fig. 5 and data not shown).

Since sterile irritants (e.g. starch) stimulate a greater macrophage response in C5-positive than in C5-negative mice,[28,29] resident macrophages represent a larger proportion of the cells in inflammatory exudates from A/J than from CBA/J mice. In order to test similar populations of cells, resident peritoneal macrophages from A/J and CBA/J mice were compared in chemotaxis ability. The macrophages had comparable kinetics of migration in response to CYAS as shown by the similar

Table 1 Time course of chemotaxis of resident macrophages to activated serum

Incubation Time (h)	Attractant[a]	No. macrophages/field[b]		Chemotactic index[c]		
		CBA/J	A/J	CBA/J	A/J	p[d]
0.5	CYAS	2.0 (1.2)	5.1 (0.1)	0.4	0.6	NS
1	CYAS	9.5 (1.1)	9.1 (2.2)	2.1	1.2	NS
2	CYAS	10.4 (1.8)	31.2 (7.5)[e]	2.3	4.0	NS
4	CYAS	43.2 (2.6)	103.5 (7.9)[f]	2.9	4.1	NS
0.5–2[g]	Diluent	4.5 (1.2)	7.9 (2.0)			
4	Diluent	14.8 (1.8)	25.2 (2.3)			

[a] Diluent (GBSS with 0.2% BSA) or a 1/100 dilution of CYAS was used.

[b] The number of macrophages that migrated through the filter after incubation for the indicated time in a Boyden chamber were counted. Data are the mean number per field (SE), obtained from 2–3 wells in each of four experiments.

[c] The chemotactic index is expressed as the mean number of macrophages per field responding to activated serum divided by the number of macrophages per field responding to diluent.

[d] Significance (ANOVA) of the CBA/J value in comparison to the comparable A/J value. NS = not significantly different.

[e] $P = 0.04$, compared to the corresponding CBA/J value.

[f] $P = 0.002$, compared to the corresponding CBA/J value.

[g] Values (No. macrophages/field) of diluent control chambers incubated for 0.5 h to 2 h were not significantly different. The values shown are the means of the controls at all three incubation times.

chemotactic indices (Table 1). Greater migration of A/J macrophages in the presence of diluent alone appeared to account for the apparent differences after 2–4 h of incubation (Table 1). Also, there were no significant differences in the responses of resident macrophages to dilutions of CYAS ranging from 1/50–1/5000 (data not shown). The responses of the resident cells were generally less than those of the elicited macrophages in the multiwell chemotaxis chamber, and appeared to account for any differences in responses of elicited cells from the mice (Fig. 5).

Since PMN are present in very small numbers in the resident peritoneal cell population (Figs 2–4),[27] elicited PMN can be compared in the two strains of mice. Chemotaxis by PMN after 1 and 3 h of incubation is shown in Table 2. At both times, A/J PMN were significantly less responsive to the complement-derived attractant than were the CBA/J PMN. These differences were observed in both the unadjusted PMN counts and in the chemotactic indices. CBA/J PMN were also more responsive to lower concentrations of attractant than were A/J PMN. A maximal response with CBA/J cells was obtained with a 1/100 dilution of CYAS, whereas 1/10 CYAS was required to induce peak chemotactic responsiveness in A/J PMN (data not shown).

(ii) Sterne-derived chemoattractant. Many bacterial species produce substances that are chemotactic for PMN.[30-35] Since large numbers of PMN appeared in exudates from both C5-positive and C5-negative mice soon after intraperitneal inoculation with viable organisms (Figs 2 and 3), the PMN chemotactic responses to a Sterne-derived factor were tested. The Sterne filtrate was chemotactic for both A/J and CBA/J PMN at dilutions of at least 1/500 (Table 3), although A/J PMN were significantly less responsive than CBA/J PMN (Table 3). Heating the Sterne filtrate abolished, and pH 2 and pH 10 treatment reduced, the macrophage cytotoxicity of the filtrate (data not shown), but did not alter the chemoattractant activity for PMN (Table 3). Untreated filtrate was suitable for testing PMN chemotactic activity, since PMN are resistant to

Table 2 Chemotactic responses of starch-elicited peritoneal PMN to activated sera

PMN[a]	Attractant[b]	No. PMN/field after[c]		Chemotactic index[d]	
		1 h	3 h	1 h	3 h
A/J	Medium	1.8 (0.6)	11.2 (2.3)	—	—
	A/J–EAMS	0.6 (0.2)	14.1 (3.3)	0.3	1.2
	CBA/J–EAMS	7.8 (1.1)	25.0 (5.2)	4.3	2.2
CBA/J	Medium	2.3 (0.9)[e]	9.1 (2.6)[e]	—	—
	A/J–EAMS	5.3 (1.0)[f]	49.3 (14.8)[e]	2.3	5.4
	CBA/J–EAMS	30.0 (3.5)[f]	119.1 (31.3)[f]	13.0	13.1

[a] Peritoneal PMN were collected 4 h after inoculation of mice with starch, and 0.5–1×10⁶ PMN suspended in the medium described by Stevenson *et al*.[29] were added to the upper wells of Boyden chambers.

[b] Attractants were the medium or sera from A/J or CBA/J mice activated with *S. typhi* endotoxin (EAMS). The EAMS attractants were used after diluting them 1/100 in medium.[29]

[c] The number of PMN that migrated through the filter after incubation for 1 h or 3 h were counted. Data are the mean number per field (SE) of three wells in each of three experiments.

[d] The chemotactic index is expressed as the mean number of PMN per field responding to activated serum divided by the number of PMN per field responding to diluent.

[e] Not significantly different, compared to corresponding A/J value.

[f] $P < 0.025$, compared to corresponding A/J value.

Table 3 Chemotactic responses of starch-elicited peritoneal PMN to Sterne bacterial factor

Attractant	Treatment	Dilution[d]	No. PMN/field[b]		P value[c]
			CBA/J	A/J	
Filtrate[a]	Untreated	1/500	8.2 (0.1)	6.5 (1.7)	NS
		1/100	16.0 (2.9)	12.8 (0.6)	NS
		1/10	38.6 (7.8)	15.7 (0.5)	0.10
	pH2	1/100	23.3 (3.6)	10.8 (1.8)	NS
		1/10	40.4 (0.4)	17.3 (2.9)	0.016
	Heated	1/100	23.4 (1.4)	16.8 (5.2)	NS
		1/10	40.7 (0.4)	22.8 (2.8)	0.02
	Combined[e]	1/100	20.9 (0.9)	13.5 (1.8)	0.02
		1/10	39.9 (2.1)	18.6 (1.7)	1.2×10^{-5}
Diluent	—	—	0.6 (0.2)	0.6 (0.2)	NS

[a] The attractant was a filter-sterilized supernatant of Sterne strain culture grown for 24 h in R medium with 0.8% bicarbonate. The chemotactic responses of PMN to untreated, heated, or pH 2 treated supernatant were tested.

[b] Peritoneal PMN were collected 4 h after inoculation of mice with starch, and 5×10⁴ were added to the wells above the filter of the multiwell chemotaxis chamber. The data are shown as the mean number per field (SE) of four wells in each of two experiments.

[c] Significance of CBA/J value compared to corresponding A/J value, by ANOVA; NS = not significantly different.

[d] The attractants were diluted in GBSS with 0.2% BSA.

[e] For each dilution of 24 h bacterial factor, the data obtained with those from untreated, heated, and pH 2 treated preparations were combined to calculate the overall mean.

killing by anthrax toxin *in vitro* up to approximately 4 h (A. Friedlander, unpublished data).

Bacterial culture fluids are also chemotactic for human and animal monocytes.[32,33,36] Heated Sterne filtrates induced a weak chemotactic response in resident mouse macrophages, and the chemotactic activity was enhanced only slightly more than was the random migration of the cells (data not shown). The chemotactic responses of A/J and CBA/J macrophages were not statistically different. Starch-elicited macrophages were not tested.

Phagocytosis and intracellular killing of Sterne spores

(i) Macrophages. Resident peritoneal macrophages from A/J and CBA/J mice were compared in their abilities to phagocytose and kill viable Sterne spores *in vitro*. First, we determined conditions for controlling growth of the extracellular bacteria in infected macrophage cultures. The minimum inhibitory concentration of gentamicin for spores was 2.5 μg/ml. For the assays, 5.0 μg of gentamicin per ml was used, a concentration which does not penetrate macrophages and contribute to intracellular bacterial killing.[37] We also performed the killing assays with rat serum, which unlike mouse serum, is bactericidal for spores of *B. anthracis*.[38] Viable counts of Sterne spores incubated in macrophage culture medium containing 10% rat serum decreased by a mean of 1.0 log after 4 h and 1.4 logs after 24 h incubation. Growth of Sterne was uninhibited in medium supplemented with rat serum which had been heated for 30 min at 56°C.

Macrophages from A/J and CBA/J mice phagocytosed spores equally well when incubated in the presence of non-immune mouse serum. After addition of spores and incubation for 60 min, between 90 and 100% of the macrophages from both strains of mice contained spores, and the phagocytic index (PI)[42] values ranged from 400 to 2000. Killing of intracellular organisms was extensive and similar for A/J and CBA/J macrophages after the 4 h incubation (Fig. 6), regardless of the reagent used to control extracellular growth. The mean reduction in the number of bacteria/macrophage in the gentamicin-containing cultures was 17.6-fold for the A/J and 15.4-fold for the CBA/J phagocytes [Fig. 6(a)]. The analogous ratios for the rat serum-containing cultures were 5.2-fold for A/J and 4.6-fold for CBA/J macrophages [Fig. 6(b)]. Neither phagocytosis nor killing was influenced by the source of nonimmune mouse serum used. Sera from A/J and CBA/J mice, and pooled normal mouse serum (Hazelton Research Products, Inc., Denver, PA) gave comparable results (data not shown).

(ii) PMN. Phagocytosis of spores by mouse peritoneal PMN was dependent on the elicitant used to obtain the PMN. PMN elicited by sterile irritants phagocytosed Sterne spores poorly (Table 4 and data not shown). In contrast, the formaldehyde-killed spores and bacilli induced PMN better able to phagocytose viable spores, as determined by the percent phagocytosis and the PI (Table 4 and data not shown). The extent of phagocytosis of spores was similar for PMN from A/J and CBA/J mice (Table 4).

KS were used in subsequent experiments to elicit PMN for *in vitro* killing experiments. First, we determined if elicited PMN from the two mouse strains differed in their content of KS, and if the presence of these KS impaired the ability of the PMN to take up and kill viable spores. The mice were injected with doses ranging from 1×10^6–5×10^8 KS and peritoneal exudates collected 4 h later. PMN from A/J mice had significantly higher PI values than PMN from CBA/J mice elicited with the same dose of KS (data not shown). The PI values of both strains varied linearly with the dose of KS by regression analysis (A/J: $y = 1.04x - 6.41$, $P \leqslant 0.0001$; CBA/J: $y = 0.91x - 5.67$, $P \leqslant 0.0001$). Correlation coefficients were 0.91 for A/J and 0.90 for CBA/J. The higher levels of KS in A/J PMN were reflected in the significantly different slopes
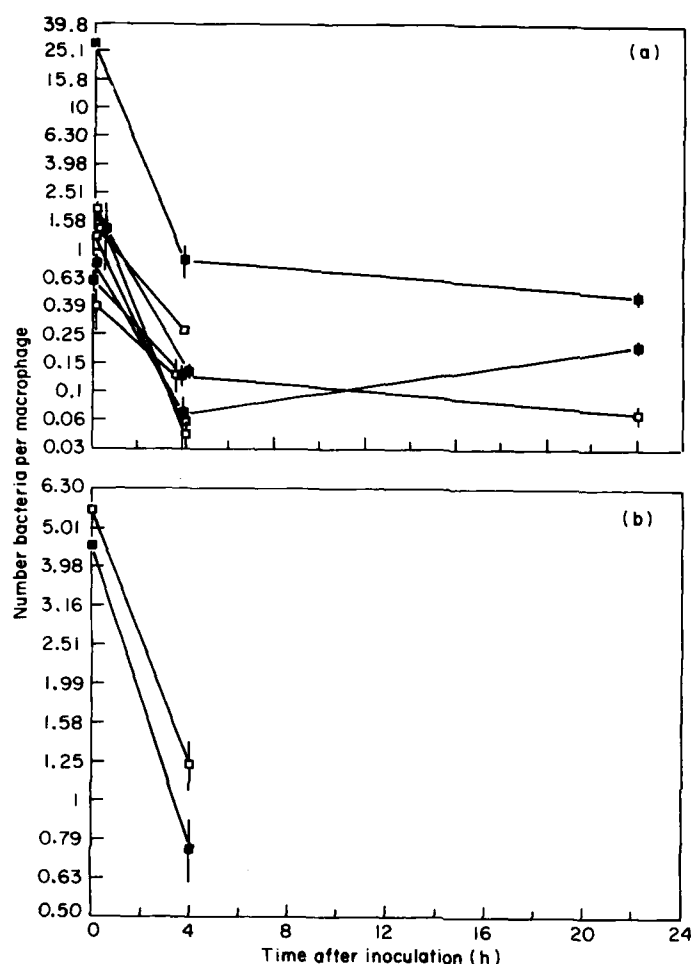
Fig. 6. *In vitro* killing of *B. anthracis* strain Sterne spores by resident peritoneal macrophages from A/J and CBA/J mice. The macrophages were infected with Sterne spores, as described in Materials and methods, lysed 0, 4 and 24 h after infection, and the lysates plated to determine the number of viable cfu. Macrophages were counted microscopically in separate wells. The data are expressed as the number of viable cell-associated bacteria/the number of macrophages. The 0 h data were determined after the 60 min phagocytosis. Growth of residual unphagocytosed organisms was inhibited by 5 μg of gentamicin per ml (a) or 10% rat serum (b). Each point represents the mean of four wells $\pm$ SE, and the results from four experiments per mouse strain (a) or one per strain (b) are shown. $\square$ = A/J mice; $\blacksquare$ = CBA/J mice.

($P = 0.001$). The differences were especially apparent at the highest doses. For example, after elicitation with 5×10^8 KS, 50% of PMN from CBA/J mice contained KS with a PI of 182.9 ($\pm$39.3), whereas 73.4% of PMN from A/J mice contained KS with a PI of 570.1 ($\pm$88.6) ($P < 0.003$).

Despite the higher burden of KS in PMN of A/J mice, these PMN phagocytosed viable spores *in vitro* as well or better than the CBA/J PMN, when elicited with $\leqslant 2.5 \times 10^8$ KS (data not shown). However, PMN from A/J mice injected with 5×10^8 KS were unable to take up additional viable spores. In contrast, 44% of the elicited PMN from CBA/J mice phagocytosed live spores, with a net PI of 621.8.

Killing of Sterne spores by A/J and CBA/J PMN was compared by using PMN elicited with three different doses of KS. PMN elicited from both mouse strains with 5×10^8 KS were unable to kill spores, however, PMN elicited with 1×10^8 KS were

Table 4 *In vitro* phagocytosis of viable Sterne spores by peritoneal PMN

Elicitant[a]	PMN	Input cfu/PMN[b]	% Phagocytosis[c]	Phagocytic index[d]
Starch	CBA/J	3/1	38.8	61.5 ± 9.1
		8/1	54.3	128.0 ± 17.3
	A/J	8/1	30.0	89.7 ± 13.5
Killed spores	CBA/J	3/1	85.8	387.3 ± 27.3
		10/1	83.0	694.0 ± 82.0
	A/J	10/1	92.0	756.5 ± 60.7

[a] Mice were injected i.p. with 1 ml volumes of 2% starch suspension or 2.5×10^8 KS. Peritoneal exudates were collected 4 h later.

[b] Determined by viable count of spore suspension and microscopic count of purified PMN suspension.

[c] Percent PMN containing at least one organism as determined by microscopic counts of organisms in PMN after incubation for 30 min at 37°C with spores, as described in the text.

[d] The percent phagocytosis multiplied by the mean number of organisms per phagocytosing PMN (mean ± SE). The data were obtained from 3–4 tubes in two experiments per elicitant. Corresponding phagocytic indices of A/J and CBA/J PMN were not significantly different.

sporicidal *in vitro*. Serum was required, but again the source of the serum (A/J or CBA/J mice) did not influence the extent of killing. The PMN were only sporicidal when they were present at a ratio of approximately 1/1 (PMN/cfu) or greater (data not shown). The kinetics of sporicidal activity were compared for A/J and CBA/J PMN at initial PMN/cfu ratios ranging from 4/1 to 30/1. The extent of killing by A/J PMN elicited with 1×10^8 KS was variable, but generally less than by CBA/J PMN to a small but significant extent (data not shown).

In contrast, PMN elicited from A/J mice with 2×10^7 KS were as sporicidal as CBA/J PMN (Fig. 7). Comparable killing was obtained regardless of the total number PMN (1×10^6 or 5×10^6) and of the input ratio of PMN/cfu used (Fig. 7). The one exception was the slightly greater killing of spores by A/J PMN in experiments having a PMN/cfu input of 27/1 [Fig. 7(b)].

Discussion and conclusions

The Sterne strain of *B. anthracis* is used as a live veterinary spore vaccine, since it is attenuated yet induces solid immunity against anthrax in laboratory and domestic animals.[1,6–9] Replication and production of anthrax toxin by Sterne are necessary to elicit effective immunity in mice.[9] C5-deficient strains of mice, such as the A/J strain, are very susceptible to killing by Sterne and develop systemic disease similar to that caused by fully virulent *B. anthracis*.[4,5] A/J mice can be protected against a lethal Sterne infection, and survive subsequent challenge with fully virulent strains, if the animals are pretreated with either normal, complement-containing serum or antitoxin antibodies.[9] These treatments appear to enhance the innate or acquired immunity of A/J mice to Sterne, respectively.[9]

C5-derived peptides generated after activation of serum complement are important chemoattractants and activators of phagocytic leukocytes.[14–19] The absence of such peptides (C5a, C5 des Arg, and C567) might be partly responsible for the enhanced susceptibility of A/J mice to Sterne. Defective inflammatory responses resulting from C5 deficiency appear to play an important role in murine susceptibility to several pathogens including *Listeria monocytogenes*,[22] *Streptococcus pneumoniae*,[25] *Pseudo-*
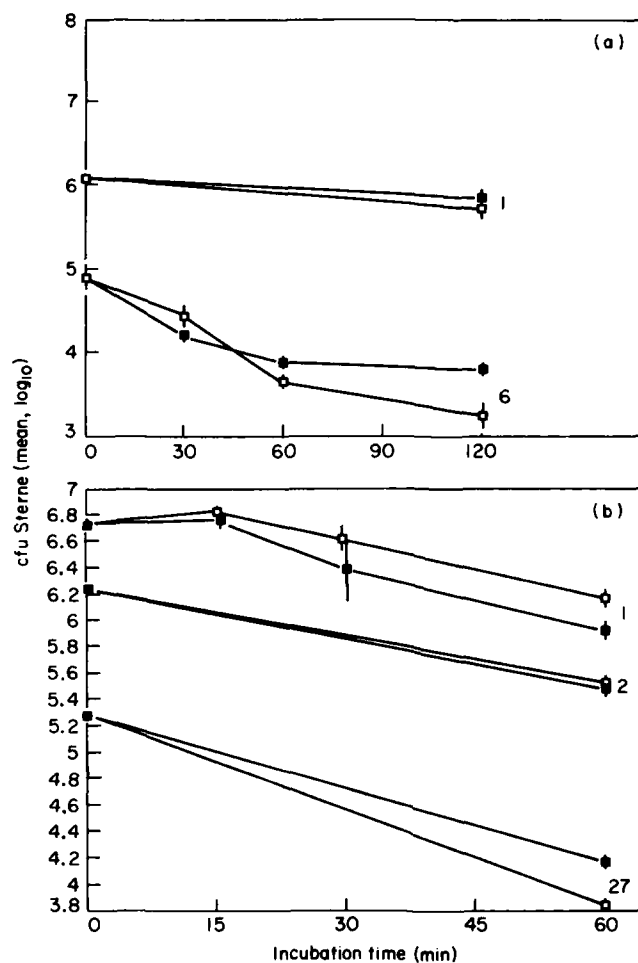
Fig. 7. Kinetics of sporicidal activity of PMN. Peritoneal PMN were elicited from A/J and CBA/J mice injected with 2×10^7 KS and combined in tubes with viable Sterne spores, as described in the text. Aliquots were lysed and plated 0, 30, 60 or 120 min after incubation. Data are shown as geometric mean ($\log_{10}$) cfu Sterne/tube (bars–SD) of triplicate tubes, and the values next to each plot are the input number PMN/cfu. Differences were significant only for data where PMN/cfu was 27 (killing by A/J PMN was greater than that by CBA/J PMN, $P = 0.005$). □, A/J mice; ■, CBA/J mice. A—1×10^6 PMN/tube. B—5×10^6 PMN/tube.

monas aeruginosa,[20,24] and *Hemophilus influenzae.*[26] The purpose of this study was to characterize quantitatively and functionally the leukocyte responses of a susceptible (A/J) and resistant (CBA/J) strain of mice to Sterne spores at the site of inoculation. These responses may be critical in host interference with initial germination or multiplication of the organism.

Intraperitoneal and subcutaneous inflammatory responses were elicited in mice to correlate these responses with susceptibility. PMN were the predominant cells present early in all elicited exudates of the mice. Macrophages were major components of later inflammatory responses to sterile irritants and live spores ($<10^8$ cfu), although macrophages responded more slowly in A/J mice than they did in CBA/J mice.

The magnitude of the intraperitoneal inflammatory responses varied with the nature of elicitant used. Sterile irritants (starch and thioglycollate) elicited significantly fewer PMN in A/J mice, compared to CBA/J mice. Similarly, Stevenson *et al.* observed a defective PMN response in A/J mice after intraperitoneal injection with thioglycollate.[39]

In vitro, A/J PMN were chemotactically less responsive than CBA/J PMN to C5-positive sera (Table 2). These data suggest that a functional defect in the cells, as well as the C5 deficiency, might contribute to the reduced *in vivo* response to inflammatory irritants.

In contrast, after an initial lag, the number of A/J PMN responding to live organisms was equal to or greater than that of CBA/J mice. Chemotactic substances other than C5-derived attractants, such as a bacterial factor or a macrophage-derived chemotactic factor for PMN could be important in recruiting PMN in A/J mice.[20,25,26,30–36,40,41] Cultures of Sterne produced a factor which effectively stimulated *in vitro* chemotaxis of PMN, although chemotaxis of the A/J cells was again significantly slower than that of CBA/J PMN (Table 3). Due to its stability and activity, the filtrate factor may be a small chemotactic peptide similar to those produced by numerous other bacteria and implicated in the recruitment of PMN and clearance of various pathogens by mice.[20,25,26,30–35]

Despite their eventual, large PMN response, A/J mice failed to contain the local proliferation and systemic invasion by the organisms. As observed previously,[4,5] A/J mice were killed by a much lower dose of spores than CBA/J mice (Fig. 2); and the strains differed in the number of germinated bacilli observed in subcutaneous tissues (Fig. 1) and in intraperitoneal exudates. Our results suggested the following possibilities concerning the role of the phagocytic leukocyte response in controlling Sterne infection: that the magnitude of the very early PMN response is important in controlling infection, that A/J PMN kill extracellular or phagocytosed spores relatively poorly, or that macrophages have a major role in the host resistance.

Phagocytosis and killing of spores by PMN were assayed with PMN from A/J and CBA/J mice elicited with killed organisms, since starch- or thioglycollate-elicited PMN phagocytosed spores poorly. The reduced sporicidal activity of A/J PMN elicited with 1×10^8 KS appeared to be due to interference of killing by residual KS within the elicited PMN, instead of to a functional defect. A/J PMN elicited with 2×10^7 KS had a lower burden of KS and killed viable spores at least as well as CBA/J cells (Fig. 7). Czuprnski *et al.* obtained similar results with PMN elicited with killed *Listeria*, and suggested that 'engorgement' of A/J PMN with dead *Listeria* hampers the listericidal activity of these cells.[42] The relative lag in mobilization of PMN could account for the higher content of KS within elicited PMN of A/J mice (Figs 3 and 4). A similar delayed accumulation of A/J PMN at the site of infection could cause a more rapid saturation of the phagocytic capacity of A/J, as compared to CBA/J, PMN. These data suggest that A/J mice have an adequate pool of PMN (or precursor cells); however, the PMN have a chemotactic defect which produces a transient lag in their response to infection.

We did not examine extracellular killing, however C5a induces PMN to secrete lysosomal enzymes that appear to have antimicrobial properties.[20,43–45] Such functions may play a role in the reduced clearance of microorganisms by PMN of C5-deficient mice.[44] Also, activated complement promotes the *in vitro* extracellular killing of some microorganisms by PMN.[43,45]

Macrophages could have a major role in the outcome of Sterne infection. The resistant CBA/J mice mobilized macrophages in response to the spores and eliminated the infection locally (Figs 1 and 2). During the same period, A/J mice responded with fewer macrophages, and a rapid proliferation of organisms took place in the tissues of the mice prior to death (Figs 1 and 2). A/J mice are known to be defective in their peritoneal macrophage response to various nonspecific stimulants.[22,23,29,39] We confirmed these findings, and showed a similar relative deficiency in their specific intraperitoneal and subcutaneous responses to viable spores and killed organisms.

Peritoneal macrophages from the A/J mice appeared to function comparably to

those of CBA/J mice in the *in vitro* assays. The chemotactic activity of resident macrophages from A/J mice was similar to that of resident CBA/J macrophages and less than that of inflammatory macrophages. The larger responses to attractant of the starch-elicited macrophages from CBA/J mice (Fig. 5) correlated with the larger proportion of resident macrophages in inflammatory exudates from A/J mice (see Results section).[28,29] Resident macrophages from A/J mice phagocytosed and killed Sterne spores as well as did those from CBA/J mice (Fig. 6). Since phagocytosis (and killing) of Sterne in the presence of nonimmune serum from A/J mice was comparable to that with sera from C5-proficient mice, C5 was not required for these functions. Similar to Sterne, susceptibility of A/J mice to *Listeria* is associated with a reduced peritoneal inflammatory response and genetically linked to the C5 deficiency.[22,29,46] Resident macrophages from A/J and *Listeria*-resistant mice (C57B1/6J and B10.A) migrate equally well and phagocytose and kill *Listeria monocytogenes* to a similar extent.[28,29,47]

Thus, our data correlate susceptibility to Sterne with the recruitment *in vivo* of an inadequate number of functional macrophages. The absence of a C5-derived chemoattractant may be directly responsible for the macrophage defect. However, A/J mice also have a relatively reduced number of monocyte precursor cells in the bone marrow.[22,23,29,48,49] The absence of a factor that stimulates stem cell proliferation, or the production of an innately low number of monocyte progenitor cells, could play a role in the poor macrophage response to Sterne.[22,23,29,48,49]

In conclusion, host susceptibility to Sterne was associated with the deficient mobilization of inflammatory cells, but not with defective uptake or killing of spores. In A/J mice, the initial lag in recruitment of PMN to the site of infection was accompanied by an inadequate macrophage response during the entire course of infection. Further studies will be done to assess the relative roles of macrophages and PMN, and the role of the protein C5, in host resistance to lethal Sterne infection.

Materials and methods

Mice. Mice were purchased from Jackson Laboratories, Bar Harbor, ME. Female mice, 7 to 12 weeks old, were used in all experiments.

(In conducting the research described in this report, we adhered to the *Guide for the Care and Use of Laboratory Animals*, as promulgated by the Committee on Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care).

Bacteria and spore preparations. The toxigenic, non-encapsulated Sterne strain of *B. anthracis* was obtained from the culture collection of the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID), Frederick, MD. Vegetative cultures of the Sterne strain were prepared by inoculation of brain heart infusion broth (Difco Laboratories, Detroit, MI) with a colony from an 18 h blood agar culture. The broth cultures were incubated 18 h at 37°C with shaking at 100 rpm. Spores were prepared from broth cultures containing at least 90% spores, as described previously.[4,5] They were further purified prior to each experiment by centrifugation on density gradients of Renografin (E. R. Squibb Co., New Brunswick, NJ). Renografin was diluted in PBS and step gradients consisting of undiluted, 40, 50 and 60% renografin made. Spore preparations were washed in PBS and centrifuged on the gradients in a Beckman SW41 rotor at 4°C, for 90 min. Ungerminated spores were collected at the interface of the 50 and 60% renografin layers; vegetative debris and germinating spores banded in the less dense layers. Spores were differentiated by Wirtz-Conklin spore stain.[50] Ungerminated spores stained with malachite green whereas vegetative debris and germinating spores stained with the safranin counterstain. The spores were washed three to four times with sterile water to remove the renografin and frozen at -70°C. Prior to injection, an aliquot of spores was thawed and suspended in phosphate-buffered saline (PBS). Dilutions were made in 0.4% (w/v) Na_2HPO_4

with 0.2% (w/v) gelatin, pH 7.0 and plated on trypticase soy agar (TSA) plates for viable counts.

Preparation of killed organisms. Killed bacterial suspensions were prepared from 18 h vegetative cultures that were washed four times with sterile PBS, and suspended to approximately 10^9 cfu per ml PBS. After removal of aliquots for viable count determination, the suspensions were autoclaved for 15 min at 121°C, or, in some experiments, incubated at 37°C with 1% formaldehyde for 24 h. Complete killing was verified by absence of growth on trypticase soy agar medium containing 5% sheep blood. The autoclaved suspensions were stored at 4°C and injected intraperitoneally into mice within 24 h. The formaldehyde-killed bacilli were washed in PBS and counted microscopically. Prior to injection of mice, the suspension was adjusted to contain $2.5–3 \times 10^8$ chains of bacilli per ml.

Spores prepared as described above were killed by incubation with 1% formaldehyde in PBS, as described by Phillips and Martin.[51] After a wash in sterile water, the spores were suspended to $\geqslant 10^9$/ml in PBS with 0.1% formaldehyde and stored at 4°C. Total counts were determined by counting a dilution in a Petroff-Hauser chamber under phase microscopy. A working suspension of the spores was prepared in PBS before each experiment and counted.

Preparation of sterile irritants. A preparation of 2% soluble starch (Difco) was made in sterile distilled water, heated to 100°C, and stirred for a few hours at 70°C until clear. The starch was stored in sterile tubes at room temperature for 2 to 3 weeks and then used undiluted. A working stock of 3% brewer's thioglycollate (Difco) was made in water and sterilized in the autoclave. A 3.5% suspension of Bio-Gel P-100 beads was prepared as previously described, and stored at 4°C.[27]

Elicitation of peritoneal inflammatory exudates. Peritoneal exudates were obtained from mice at intervals after intraperitoneal injection of 1.0 ml of either the starch, thioglycollate, KB, or KS. The mice were killed by CO_2 asphyxiation and injected i.p. with 3 ml of air and 7 ml of Ca^{2+}- and Mg^{2+}-free Hank's balanced salts solution (HBSS) with 5 to 10 units of heparin per ml. The fluid was withdrawn and the total cell count determined with a hemocytometer. Differential counts were performed on samples centrifuged in a Cytospin centrifuge (Shandon Inc., Pittsburgh, PA) and stained with Diff-Quik (Harleco, Philadelphia, PA).

Viable spores were suspended in PBS, and mice were injected i.p. with $2–3 \times 10^6$, 6×10^6, or 10^8 cfu. At intervals after infection, peritoneal exudates were harvested from individual mice as described above, except mice were injected with 5.0 ml of HBSS/heparin. Peritoneal cells were counted and differentiated, and 10 μl aliquots of undiluted sample were spread on slides over a 20 mm^2 area. Direct microscopic counts of organisms were done by the method of Holdeman *et al.*[52] Duplicate slides were stained with Diff-Quik, to detect bacilli, and with spore stain, to count spores. The number of extracellular organisms (i.e. not phagocytosed or cell-associated) was counted. Fifty fields were counted per slide to determine the mean number of bacilli and spores per specimen; the limit of detection was 7×10^5 organisms per specimen.

Elicitation of subcutaneous inflammatory exudates. Mice were inoculated subcutaneously with Bio-Gel beads or with KS. Inflammatory cells were harvested from mice 5 days after subcutaneous inoculation with Bio-Gel beads, according to the method described by Singh *et al.*[53] After filtration of the exudates to remove the beads, cells were counted and differentiated as described for peritoneal exudates. Subcutaneous pockets were prepared in mice by the method of Fauve *et al.*[27] The pockets were inoculated with 0.2 ml of KS diluted in HBSS with 5% gelatin to 2×10^7 spores per dose.[54] Four days after inoculation, the subcutaneous exudates were collected and washed with HBSS as described previously.[27,53]

Subcutaneous infection and tissue collection. Mice were inoculated subcutaneously in the upper left foreleg with 0.2 ml of renografin gradient-purified spores or PBS. At intervals from 0 to 72 h post-inoculation, the mice were killed as described[5] and the entire left foreleg removed by dissecting the leg away from the thoracic wall. The specimens were fixed in 10% buffered formalin (modified Millonig formula; Columbia Diagnostics, Inc., Springfield, VA). Transverse sections through the foreleg were obtained at the level of the shoulder and elbow joints. The specimens were embedded in paraffin, cut at 4 μm, and stained with hematoxylin and eosin (HE), Giemsa, or with spore stain. Spore stains were made by deparaffinizing and rehydrating the sections, steaming the slides for 30 min with malachite green and counterstaining for 2 min with safranin.[50] Slides were examined microscopically for the presence of spores (spore

stain) and germinated bacilli (Giemsa stain). The character and severity of the inflammatory response were evaluated with HE stain.

Preparation of peritoneal cells for in vitro *assays.* Resident macrophages were obtained by peritoneal lavage of untreated mice with heparinized HBSS, differentiated, and enumerated as described above. For chemotaxis assays, the suspensions were further purified by centrifugation in Ficoll-sodium diatrizoate leukocyte separation medium (Histopaque-1077, Sigma Chemical Co., St. Louis, MO), by the procedure recommended by the supplier. The mononuclear cells were washed twice in HBSS and resuspended in the assay media indicated below. The Ficoll-purified suspensions contained >75% macrophages.

Inflammatory macrophages were obtained from peritoneal exudates of mice injected 4.5–5 days previously with starch. They were further purified by centrifugation in leukocyte separation medium, and the resulting suspensions contained ⩾70% macrophages and <15% PMN.

Inflammatory PMN were collected from peritoneal exudates of mice injected 4 h previously with starch, thioglycollate, KS, or KB. PMN suspensions were differentiated and purified by centrifugation in leukocyte separation medium if they contained <80% PMN (chemotaxis assays) or <85% PMN (phagocytosis and sporicidal activity assays).

Preparation of chemotactic attractants. Chemotactic complement components were generated from mouse serum activated with yeast or endotoxin. Yeast-activated serum was obtained by treating pooled sera from A/J (AYAS) or CBA/J (CYAS) mice with 20 mg of yeast cells per ml by the method of Vallota and Muller-Eberhard.[55] Prior to addition to the sera, the yeast were boiled as described by Leonard and Skeel.[56] Unused aliquots of yeast were stored at −20°C. The sera were incubated with the yeast at 37°C for 50 min, and the yeast removed by centrifugation for 20 min at $300 \times g$. The sera were heated at 56°C for 30 min prior to storage in aliquots at −70°C. Endotoxin-activated mouse sera from A/J and CBA/J mice were prepared by the method of Boetcher and Meltzer,[57] and aliquots of EAMS were stored at −70°C until used.

Sterne-derived chemoattractant was produced by a modification of the method of Rot *et al.*[36] Synthetic R medium with 0.8% yeast extract and 0.8% bicarbonate,[58] was inoculated with a suspension of fresh colonies of Sterne. The culture was incubated at 37°C with shaking at 100 rpm for 24 h. Bacteria were removed by centrifugation at 7000 rpm for 25 min (DuPont Sorvall GSA rotor), and the supernatant sterilized by filtration through a 0.2 μm filter. Aliquots were stored at −20°C until used in chemotaxis assays or tested for stability. Thermal and pH stabilities of the chemotactic activity in the supernatant preparation were tested on aliquots, as described previously.[36]

Macrophage cytotoxicity assay. Bacterial culture supernatants were tested for the presence of anthrax toxin by their cytotoxic effect on murine macrophages.[59] The colorimetric tetrazolium reduction assay of Mosmann,[60] modified as described by Green *et al.*,[61] and the J774 cell line, were used.

In vitro *chemotaxis assays.* Chemotactic activity (directed migration) of attractant was assayed by counting phagocytes migrating in response to positive gradients (i.e. larger concentration of attractant present in bottom well). Chemokinesis (enhancement of random migration)[33] of cells was tested by adding attractant only to the upper wells (with the cells), or in equal concentrations to both wells.

For Boyden chamber migration assays, we used a modification of previously described methods.[29,57] Attractant (100 μl) diluted in the medium described by Stevenson *et al.*[29] or in Gey's balanced salts solution (GBSS) with 0.2% bovine serum albumin (Sigma, BSA) was added to the bottom well of Neuro Probe blind well chambers (Nucleopore Corp., Pleasanton, CA). Polycarbonate chemotaxis filters (Nucleopore) were added and cell suspensions (100 μl) were placed in the top well. PMN were suspended in medium,[29] 0.5–1 × 10⁶ added per chamber, and chambers were incubated 30 min to 3 h. Macrophages were suspended in the medium of Stevenson *et al.*[29] or in GBSS with 2% BSA, 0.5–1 × 10⁶ per chamber were added, and chambers were incubated from 30 min to 4 h. Results were enumerated as described.[29] The chemotactic index was expressed as the mean of the number of cells per field responding to activated serum, divided by the number of cells per field responding to diluent.

Migration assays were also done using a 48-well micro chemotaxis assembly (multiwell chamber) as specified by the manufacturer (Neuro Probe, Inc., Bethesda, MD). The methods originally described by Harvath *et al.*[62] for PMN and by Falk *et al.*[63] for monocytes were

followed, except that mouse peritoneal cells (5×10^4 per well) were used instead of human peripheral blood loukocytes. The chamber was incubated in humidified air with 5% CO_2 at 37°C, for 1 h (PMN) or 2 h (macrophages).

Phagocytosis. Phagocytosis of viable spores by peritoneal PMN was determined by the procedure of Czuprynski,[42] with the following changes. Each tube contained 2×10^6 PMN, 2.5×10^7 cfu spores, 10% pooled mouse serum, and HBSS in a total volume of 0.5 ml. The tubes were incubated at 37°C horizontally with shaking at 140 rpm for 30 or 60 min. The phagocytes were washed four times in HBSS with 0.1% gelatin and then stained with Wirtz-Conklin spore stain[50] and with Diff-Quik stain. The percent of phagocytosing PMN and the number of organisms per phagocytosing PMN were determined by light microscopy.[42] The PI was calculated: (% PMN containing $\geqslant 1$ organism (spore or bacillus) $\times$ the number of organisms per PMN).[42]

Resident macrophages were added to wells of 24-well culture plates to which 13 mm diameter sterile cover slips had been added.[37] After addition of spores and incubation for 60 min, the coverslips were washed three times with HBSS, fixed in methanol, stained as described for PMN, and the PI determined.[28,42]

In vitro sporicidal assays. Peritoneal PMN elicited with KS were assayed for sporicidal activity by a method similar to previously described procedures.[42,64] In most experiments, the following reactants were added in triplicate to 12×75 mm tubes in a total volume of 0.5 ml: (1) 10% mouse serum, (2) HBSS, (3) 1×10^6 PMN, and (4) renografin-purified Sterne spores. The latter were diluted in HBSS and added to obtain a PMN/cfu ratio of $0.1/1-30/1$. In some experiments, the total reaction volume was 1.0 ml, and 5×10^6 PMN were used. The mouse serum used was a pool of sera from outbred, nonimmune mice (Hazelton Laboratories, Rockville, MD) or pools collected from A/J or CBA/J mice. The assay tubes were capped tightly, sealed with Parafilm, and either incubated horizontally at 37°C with shaking at 140 rpm, or rotated at 37°C at 8 rpm. Killing was not affected by either incubation procedure. Sporicidal activity was assayed immediately and after incubation for 30, 60 or 120 min. The tubes were put in an ice-water bath to stop the reaction, and a 0.1 ml aliquot was added to 0.9 ml of lysing solution (0.01% BSA in distilled water). After incubation for 5 min at room temperature, the lysed sample was diluted in 0.4% (w/v) Na_2HPO_4 with 0.2% (w/v) gelatin, pH 7.0. Dilutions were plated on trypticase soy agar (Difco) medium for viable count determinations.

Macrophage killing of spores was assayed by a modification of the procedure described by Lissner et al.[37,65] Resident peritoneal macrophages from A/J and CBA/J mice were cultured *in vitro*,[37] and infected with purified spores at an input multiplicity of 10 to 33 cfu per macrophage (10 to 20 in most experiments). After incubation for 60 min, the macrophage cultures were washed, 5 μg of gentamicin per ml or 10% rat serum added, and cultures were incubated for 0, 4 or 24 h at 37°C in humidified air with 5% CO_2. Killing of intracellular organisms was determined as described above for PMN. At each time point, the number of adherent macrophages was determined in uninfected wells, according to the nuclear staining procedure of Nakagawara and Nathan.[66]

Statistics. Data were analysed by analysis of variance (ANOVA) with Fisher's least significant difference test, t-test, or linear regression, as indicated in the text. Computer programs used included the Statistical Analysis System, SAS Institute, Cary, NC; the Computerized Biostatistical Analysis Library, USAMRIID; Epistat statistical programs, provided by T. Gustafson, Round Rock, TX; and Stat-Packets, Walonick Associates, Inc., Minneapolis, MN.

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